



Regular Article

High thrombin activatable fibrinolysis inhibitor levels are associated with an increased risk of premature peripheral arterial disease

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ABSTRACT

Background: Previous studies suggested that hypofibrinolysis is associated with increased risk of peripheral arterial disease. Thrombin activatable fibrinolysis inhibitor (TAFI) has been identified as an important inhibitor of fibrinolysis. The aim of our study was to assess the role of TAFI in young patients with peripheral arterial disease.

Methods: In a single-center case-control study we measured plasma TAFI antigen levels and functional TAFI in consecutive young patients (men 18–45 years and women 18–55 years) with a first manifestation of peripheral arterial disease and compared these with a population-based control group.

Results: A total of 47 peripheral arterial disease patients and 141 controls (mean age 43) were included. Intact TAFI antigen levels were significantly higher in patients with peripheral arterial disease ($112.4 \pm 21.1\%$) than in controls ($104.9 \pm 19.9\%$, $p = 0.03$). The risk of peripheral arterial disease increased with 18% (OR 1.18; CI 1.01–1.34) per 10% increase of TAFI antigen. Functional TAFI levels were slightly higher in patients compared to controls, however this difference was not significant. For individuals with the highest functional TAFI levels, above the 90th percentile, the increased risk for peripheral arterial disease was most pronounced (OR 3.1; CI 1.02–9.41).

Conclusion: High TAFI levels are associated with increased risk of premature peripheral arterial disease.

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Introduction

Previous studies have reported associations between hypofibrinolysis and an increased risk of peripheral arterial disease (PAD) [1,2]. Most studies on hypofibrinolysis focussed on elevated plasminogen activator inhibitor-1 (PAI-1) levels and showed association between PAI-1 and PAD [3,4] or symptoms of PAD [5]. Also the functional PAI-1 -675 (4 G/5 G) polymorphism was associated with early thrombotic reclosures [6].

Thrombin activatable fibrinolysis inhibitor (TAFI) has also been identified as an important inhibitor of fibrinolysis [7,8]. Activated TAFI (TAFIa) exerts its anti-fibrinolytic function by removing the carboxy-terminal lysine residues of partially degraded fibrin. This results in a decreased binding and activation of plasminogen and thereby attenuates fibrinolysis [9,10]. TAFI is activated by thrombin, plasmin or the thrombin/thrombomodulin complex by a single cleavage at Arg-

92. This results in the release of the activation peptide (TAFI-AP) and exposure of the substrate binding site of TAFIa. TAFIa is thermolabile due to a spontaneous conformational conversion into an inactive form (TAFIa(i)), which is sensitive to further proteolytic cleavage.

Several studies have been performed to study TAFI levels in arterial thrombosis. It has been shown that increased functional TAFI levels and/or TAFI antigen levels are associated with ischemic stroke [11,12]. The relationship between TAFI levels and the risk of coronary heart disease is less clear, since several conflicting studies have been reported [13–15]. So far no studies have been performed to assess the role of TAFI in peripheral arterial disease.

Therefore, the aim of our study was to investigate both antigen and functional TAFI-levels, including the activation markers TAFI-AP and TAFIa(i) in young patients with a first clinical manifestation of peripheral arterial disease.

Patients

The “Genetic risk factors for Arterial Thrombosis at young age: the role of TAFI and other Coagulation factors (ATTAC)” study is a single-center, case-control study to explore the role of TAFI levels and the

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incidence of arterial thrombosis at young age. Cases included in this study were consecutively recruited patients with a first-ever ischemic event due to peripheral arterial disease at the department of Vascular Surgery of the Erasmus Medical Center Rotterdam in the Netherlands. The diagnosis PAD was defined as peripheral arterial stenosis resulting in ischemia, classified according to the Rutherford criteria [16]. Patients were eligible for inclusion if they were 18–45 years for males and 18–55 years for females. Patients were included one to three months after the event in order to avoid a possible influence of the event or an acute phase response on plasma levels of TAFI and TAFI activity.

A control group was obtained by asking the patients to bring a friend, neighbour or partner fulfilling the same age criteria but without a history of arterial thrombosis. Relatives of patients were not permitted. A detailed clinical history and physical examination, including arterial pulsations of upper and lower extremities, was performed in both patients and controls. For this study, 3 age- and sex-matched controls were randomly selected for each patient. Information was obtained on cardiovascular risk factors, including smoking, hypercholesterolemia, diabetes mellitus, hypertension and family history of cardiovascular disease. Smoking status was defined as never, previous or current smoker. Hypercholesterolemia was defined as total cholesterol level >5.0 mmol/l or receiving lipid-lowering treatment on day of the ischemic event. Patients with a medical history of diabetes or using either oral anti-diabetic medication or insulin therapy on day of the event were considered to be diabetics. Hypertension was defined by a systolic blood pressure ≥ 140 mm Hg, and/or diastolic blood pressure ≥ 90 mmHg or the use of anti-hypertensive drugs. A positive family history was noted if the patient had a first-degree relative with a history of cardiovascular disease before the age of 60.

Written informed consent was obtained on enrollment from each participant, and the study was approved by the Medical Ethics Committee of Erasmus MC and conducted according to the procedures of the Declaration of Helsinki.

Methods

Blood was drawn under strictly standardized conditions [17], by venipuncture in the antecubital vein using the Vacutainer system (Becton Dickinson, Plymouth, UK). Blood for coagulation studies in both patients and controls was collected in 3.2% trisodium citrate (9:1 vol/vol). Citrated blood was centrifuged within one hour at 2,000 g for 10 min at 4 °C. Plasma was additionally centrifuged at 20,000 g for 10 min at 4 °C and stored at -80 °C until analysis.

TAFI antigen levels

Three recently developed sandwich-type ELISAs (i.e. MA-T12D11/MA-T30E5-HRP, MA-T12D11/MA-T18A8-HRP and MA-T30E5/MA-17D7-HRP) were used to quantify the plasma levels of TAFI, TAFI activation peptide (TAFI-AP) and (in)activated TAFI (TAFIa(i)), respectively [18]. To obtain pooled human plasma, blood samples ($n=21$ blood donors) were collected in 4% citrate according to the guidelines of the blood transfusion centre (Red Cross, Leuven, Belgium) and plasma was pooled. Either non-activated pooled human plasma (MA-T12D11/MA-T30E5-HRP ELISA) or activated pooled human plasma (MA-T12D11/MA-T18A8-HRP and MA-T30E5/MA-17D7-HRP ELISAs) was used as a standard (1:40 dilution of plasma in PTAE buffer (PBS pH 7.4 containing 0.002% Tween 80, 1 g/l BSA and 5 mM EDTA), followed by serial two-fold dilutions up to 1:2560). For preparation of the standard for the MA-T12D11/MA-T18A8-HRP and MA-T30E5/MA-17D7-HRP ELISAs, pooled human plasma was incubated with thrombin (20 nM), thrombomodulin (5 nM) and CaCl_2 (17 mM) in Hepes buffer at 37 °C for 15 minutes. The reaction was

stopped by addition of H-D-Phe-L-Prolyl-L-arginine chloromethylketone (PPACK, 30 μM final concentration).

Plasma samples derived from patients were diluted 1:160 (MA-T12D11/MA-T30E5-HRP ELISA), 1:80 (MA-T12D11/MA-T18A8-HRP ELISA) and 1:80 (MA-T30E5/MA-17D7-HRP ELISA). All values of TAFI and TAFI activation markers are expressed relative to the levels in the pooled human plasma.

The assay variability of the ELISAs was evaluated using 4 different plasma samples each assayed 4 times on 4 occasions. The intra-assay and inter-assay coefficients of variation for MA-T12D11/MA-T30E5-HRP were 6.2% and 8.3%, respectively, for MA-T12D11/MA-T18A8-HRP 3.1% and 7.3%, respectively and for MA-T30E5/MA-T17D7-HRP 3.3% and 6.4%, respectively.

The interdilution coefficients of variation were 8.2%, 5.1% and 7.3% for MA-T12D11/MA-T30E5-HRP, MA-T12D11/MA-T18A8-HRP and MA-T30E5/MA-T17D7-HRP, respectively. The detection limit of all three ELISAs was 1.6%. TAFI-depleted plasma revealed no detectable response in any ELISA.

TAFI activity assay

The TAFI functional assay was performed essentially as described elsewhere [19]. Briefly, 100 μL of diluted plasma samples (20-fold dilution of individual plasmas in TAFI-depleted pooled normal plasma) were added to the wells of a microtitre plate containing 25 μL of a reaction mix composed of thrombin, Solulin (recombinant thrombomodulin which was a gift from PAION GmbH, Aachen, Germany (by courtesy of Dr. H. Brohmann)), CaCl_2 , and recombinant tissue plasminogen activator (tPA) (Actilyse) (final concentrations in the assay: 3.3 NIH units mL^{-1} , 2.0 $\mu\text{g}/\text{mL}$, 20 mM, 20 mM, and 0.10 $\mu\text{g}/\text{mL}$, respectively), forming a plasma clot. A control with addition of potato carboxypeptidase inhibitor (PCI) was performed for each sample (final concentration 30 $\mu\text{g}/\text{mL}$). The wells were immediately covered with paraffin oil and the plate was placed in a pre-warmed (37 °C) incubation chamber of a microplate reader (Victor³™ multilabel counter, Perkin Elmer, Turku, Finland). The optical density at 405 nm was monitored for 150 min. Lysis time (LT) was defined as the time-point corresponding to a 50% decrease in optical density. Functional TAFI levels were calculated as TAFI-related retardation (RT), defined as the difference between the LT in the absence and in the presence of PCI ($\text{LT}_{\text{PCI}} - \text{LT}_{+\text{PCI}}$).

Using this assay the intra- and inter-assay variability of the TAFI-related retardation of pooled normal plasma (20-fold diluted) were 7% and 13%, respectively ($n=30$; using several batches of TAFI-depleted plasma) [19]. No influence was detected of the use of oral anticoagulant therapy with vitamin K antagonists on functional TAFI assay (data not shown).

Fibrinogen, CRP and vWF

Fibrinogen levels were determined according to the prothrombin (PT)-derived method (Dade Thrombin Reagent, Siemens Diagnostics, Leusden, The Netherlands) on a Sysmex CA-1500 automated coagulation analyzer (Siemens Diagnostics, Leusden, the Netherlands). When the PT or APTT was prolonged in patients, for instance due to the use of oral anticoagulants, fibrinogen concentrations were determined according to the von Clauss method on an ACL-300 [HemosIL (Fibrinogen-C); Instrumentation Laboratory, Breda, the Netherlands]. CRP levels were measured by means of an in-house high-sensitive ELISA with polyclonal rat anti-human CRP antibodies (DAKO, Glostrup, Denmark) and a CRP-calibrator (DAKO). VWF antigen (vWF:Ag) was determined with an in-house ELISA with polyclonal rabbit anti-human vWF antibodies and horseradish peroxidase conjugated anti-human vWF (DakoCytomation, Glostrup, Denmark) for catching and tagging, respectively. The intra-assay variation coefficient for vWF:Ag was 5.7%.

Statistical analysis

The data are presented as means and standard deviations (SD) for continuous variables and as counts and percentages for categorical variables. To compare the plasma levels of TAFI variables between the controls and the PAD patients an analysis of variance (ANOVA) was used, with adjustment for age and gender. Spearman's correlation coefficient was calculated to study the associations between the various TAFI antigen levels. The association between TAFI antigen levels and PAD was investigated by logistic regression with TAFI antigen as a continuous variable, with adjustment for age and gender. In addition, we used a cut-off approach, in which the risk of PAD in patients with high TAFI levels (top 10% based on distribution of controls) was compared with risk of PAD in the remaining 90% of the population, with adjustment for age and gender. A value of $p < 0.05$ was considered statistically significant. All statistical analyses were performed using the Statistical Package for Social Science for windows, version 16.0 (SPSS Inc., Chicago, IL, USA).

Results

The patient group consisted of 47 patients with a mean age of 43.2 ± 7.9 years (range 21–55 years). The characteristics of the patients and of the 141 individuals who served as a population-based control group are summarized in Table 1. Most patients were current or previous smokers (93.6%) and had a positive family history of arterial thrombosis (63.8%) as well as other traditional risk factors. BMI in patients was not significantly higher in patients ($25.8 \pm 4.8 \text{ Kg.m}^{-2}$) than in controls ($25.5 \pm 4.5 \text{ Kg.m}^{-2}$). All patients had objectively diagnosed PAD and could be subclassified according to the Rutherford criteria: 27 (57.4%) patients with claudication (grade 2), 15 (31.9%) patients with ischemic rest pain (grade 3) and 5 (10.6%) patients with tissue loss (grade 4).

Intact TAFI antigen levels were significantly higher in patients with PAD ($112.4 \pm 21.1\%$; mean \pm SD) versus healthy controls ($104.9 \pm 19.9\%$; $p = 0.03$) (Fig. 1). The risk of PAD increased with 18% (OR 1.18; CI 1.01–1.35) per 10% increase of TAFI antigen. TAFI activation peptide, a marker for TAFI activation *in vivo*, was not significantly different from the controls, nor were the levels of activated and inactivated TAFI (TAFI a(i)) (Table 2).

Table 1
Characteristics of PAD patients and healthy controls.

	Patients (n = 47)	Controls (n = 141)	p value
Demographics			
Age – years			
Mean	43.2 ± 7.9	43.0 ± 7.6	0.85
Male sex – n (%)	13 (27.7)	39 (27.7)	0.94
Oral anticoagulant use – n (%)	13 (27.7)	2 (1.4)	<0.0001
Risk Factors			
Family history of premature cardiovascular disease – n (%)	30 (63.8)	47 (33.3)	<0.0001
Hypercholesterolemia – n (%)	22 (46.8)	6 (4.3)	<0.0001
Hypertension – n (%)	18 (38.3)	10 (7.1)	<0.0001
Diabetes mellitus – n (%)	10 (21.3)	1 (0.7)	0.001
Smoking status – n (%)			
Never smoked	3 (6.4)	60 (42.6)	<0.0001
Former smoker	21 (44.7)	46 (32.6)	<0.0001
Current smoker	23 (48.9)	35 (24.8)	<0.0001
Clinical aspects			
Body-mass index (kg/m^2)	25.8 ± 4.8	25.5 ± 4.5	NS
Rutherford criteria – n (%)			
Asymptomatic (Grade 1)	0	141	
Claudication (Grade 2)	27 (57.4)	-	
Ischemic rest pain (Grade 3)	15 (31.9)	-	
Tissue loss (Grade 4)	5 (10.6)	-	

NS = not significant.

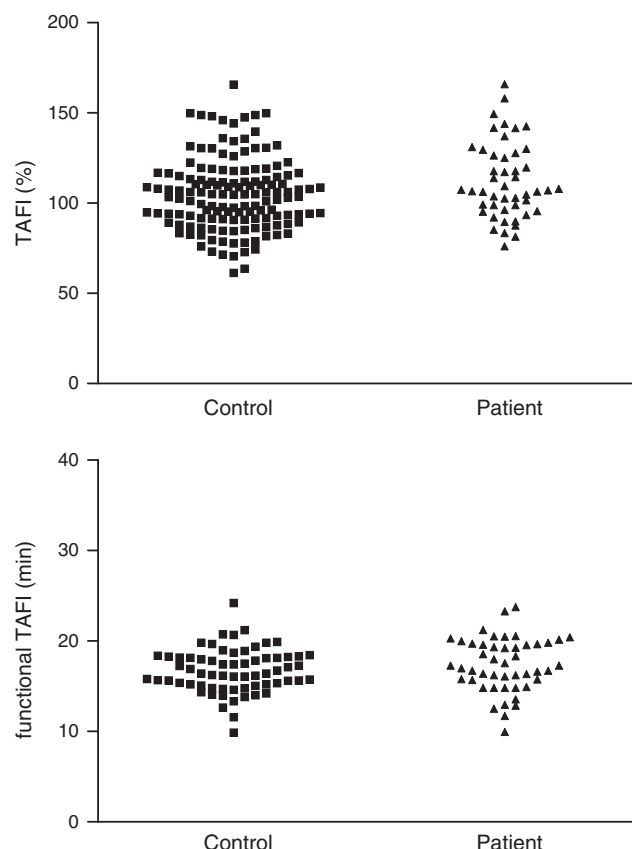


Fig. 1. TAFI antigen levels and functional TAFI levels in patients with PAD and healthy controls.

Functional TAFI was measured as TAFI-related retardation in a plasma clot lysis based assay. Functional TAFI was not significantly higher in patients compared to controls (17.4 ± 3.0 min vs. 16.7 ± 2.4 min; $p = 0.28$, respectively) (Fig. 1). To further investigate whether increased functional TAFI levels are a risk factor for PAD we calculated odds ratios using different cut-off percentiles of functional TAFI, based on the levels in the control group. Individuals with functional TAFI levels in the highest 10% percentile showed an increased risk of PAD compared to individuals in the lowest 90% percentile (OR 3.1; 95% CI 1.02–9.41).

No relationships between TAFI activation peptide and TAFI a(i) with PAD were observed in our study (Table 2). Functional TAFI was significantly correlated with TAFI activation peptide ($r = 0.19$, $p = 0.047$), but not with TAFI antigen levels and TAFIa(i) (Table 3).

Table 2
Laboratory parameters in PAD patients and healthy controls.

	Patients (n = 47)	Controls (n = 141)	p value
TAFI parameters			
TAFI antigen (%)	112.4 ± 21.1	104.9 ± 19.9	0.03
TAFI activation peptide (%)	140.9 ± 38.0	145.3 ± 52.5	0.58
TAFI a(i) (%)	139.3 ± 36.3	137.1 ± 34.2	0.71
Functional TAFI [TAFI-related retardation] (min)*	17.4 ± 3.0	16.7 ± 2.4	0.28
Haemostatic/inflammatory parameters			
Fibrinogen (g/L)	4.07 ± 0.85	3.39 ± 0.96	<0.001
vWF:Ag (U/mL)	1.47 ± 0.52	1.03 ± 0.38	<0.001
hsCRP (mg/L)	3.29 ± 4.58	1.56 ± 3.58	0.006

* The TAFI functional assay was measured as TAFI-related retardation and expressed in minutes (mean \pm SD).

Table 3

Relationship between the various TAFI antigen levels in PAD patients and healthy control individuals.

	TAFI Ag	TAFI AP	TAFI a(i)	TAFI-related retardation	Fibrinogen	vWF: Ag	hsCRP
TAFI Ag		0.39	0.15	0.16	0.17	0.13	0.06
p value		<0.0001	0.04	0.10	0.07	0.07	0.43
TAFI AP			0.26	0.19	0.05	0.03	0.05
p value			<0.0001	<0.05	0.61	0.67	0.50
TAFI a(i)				-0.02	-0.09	0.05	-0.01
p value				0.80	0.32	0.50	0.93
TAFI-related retardation					0.17	0.10	0.17
p value					0.07	0.30	0.08

Correlations (non-parametric, Spearman 2-tailed).

The strongest correlation was seen between TAFI antigen levels and TAFI activation peptide levels ($r = 0.39$, $p < 0.0001$) and between TAFI activation peptide levels and TAFI a(i) ($r = 0.26$, $p < 0.0001$).

Fibrinogen, hsCRP and vWF antigen levels were significant higher in patients with PAD versus healthy controls. Mean fibrinogen levels were 4.07 ± 0.85 g/L in PAD patients versus 3.39 ± 0.96 g/L ($p < 0.001$) in healthy controls. Mean hsCRP levels were 3.29 ± 4.58 mg/L in PAD patients versus 1.56 ± 3.58 mg/L ($p = 0.006$) in healthy controls. Mean vWF:Ag levels were 1.47 ± 0.52 U/ml in PAD patients versus 1.03 ± 0.38 U/ml ($p < 0.001$) in healthy controls.

Discussion

We studied for the first time TAFI levels in a well defined group of patients with peripheral arterial disease at young age. We showed that high TAFI levels are associated with increased risk of PAD. This is in line with previous reports that increased TAFI levels are associated with other forms of arterial thrombosis, i.e. ischemic stroke [11,12].

Previous studies have indicated that hypofibrinolysis may be associated with increased risk of arterial thrombosis including coronary heart disease, ischemic stroke and PAD, although this was not confirmed in all studies [3,4,20–25]. This increased risk has mainly been attributed to increased levels of plasminogen activator inhibitor-1 [4]. In addition, it has been suggested that the PAI-1 -675(4 G/5 G) polymorphism is associated with the outcome of PAD [6].

TAFI is an important inhibitor of fibrinolysis, by cleaving the lysine binding sites of partially degraded fibrin, which interferes with the interaction of plasminogen with fibrin. High TAFI levels may therefore result in a decreased fibrinolytic activity [9,10]. Several studies have been performed to assess the relationship between TAFI levels and the risk of arterial thrombosis. In previous studies, we and others have found that functional TAFI levels are significantly associated with the risk of first ischemic stroke [11,26]. Also TAFI antigen levels have been associated with ischemic stroke, (unstable) angina pectoris and acute myocardial infarction [11,13,27–29]. Previous studies were hampered by the fact that most antigen assays are highly sensitive to the TAFI genotype, which influences the outcome of some of the early studies (reviewed in [22]). Recently, we have developed genotype-independent TAFI antigen assays, and we were able to analyze TAFI antigen levels using three newly developed immunological assays with distinct reactivities towards TAFI and TAFI fragments [18]. These assays measure intact TAFI, the released activation peptide (TAFI-AP) and the total of activated and inactivated TAFI. So far these assays have only been used to study TAFI levels in acute ischemic stroke, coronary heart disease and severe meningococcal infection [12,15,30,31]. In acute ischemic stroke, it was shown that both intact TAFI and the activation peptide were increased in patients compared to healthy controls [12]. We previously showed that TAFIa(i) levels were higher in young patients with cardiovascular disease in our ATTAC study

[15]. In patients with coronary artery disease in the AtheroGene study the amount of activated TAFI, measured by TAFIa/TAFIai ELISA was independently associated with increased risk of cardiovascular death [30]. In severe meningococcal infection in children intact TAFI and the activation peptide were also significantly increased [31].

We have studied young patients with PAD, because it is suggested that in these young patients other pathogenetic mechanisms than the classical risk factors may be of importance [4,32]. Patients were included one to three months after the first ischemic event to exclude an acute phase response, although previous studies have suggested that the relationship between TAFI levels and arterial thrombosis is both seen in the acute phase and at three-months follow-up [11,12]. In our study, TAFI antigen levels were significantly higher in PAD patients than in the healthy controls. TAFI-AP levels and TAFIa(i) levels were not higher in PAD patients. Since TAFI-AP is a marker of the extent of TAFI activation, this suggests that TAFI activation is not systemically increased in PAD patients.

We measured functional TAFI levels using a previously described clot lysis based method. By measuring clot lysis time with and without a specific inhibitor of TAFI (PCI) the functional activity of TAFI can be determined. Furthermore, a clot lysis based assay is a more physiological method than assays using chromogenic substrates to study the impact of TAFI on fibrinolysis [19]. A trend was seen for higher functional TAFI activity in PAD patients compared to controls, but this did not reach statistical significance. Individuals with functional TAFI levels above the 90th percentile however had an increased risk of PAD.

We also studied other haemostatic variables (Fibrinogen and vWF:Ag levels) and a marker of inflammation (hsCRP), which are of importance as risk factors in the development of PAD [33], in relation to TAFI. Fibrinogen, hsCRP and vWF were all significantly elevated in PAD patients in comparison to the healthy controls. However, the correlation between TAFI antigen with fibrinogen, vWF Ag and CRP was very weak. This suggests that the association between TAFI and PAD is independent of an inflammatory status or endothelium damage. Prospective studies are needed to investigate the association between TAFI levels and cardiovascular disease as, on the one hand, high TAFI levels may facilitate the development of cardiovascular comorbidity by shifting the haemostatic balance to a more hypofibrinolytic state and, on the other hand, TAFI levels may relate to the body's mechanisms dampening the excessive inflammatory reaction, such as previously found in patients with rheumatoid arthritis [34,35]. Growing evidence suggests that inflammation, oxidative stress and hypofibrinolysis may have a pivotal role in the high prevalence of cardiovascular disease [36].

Our study has some important strengths. We measured TAFI antigen levels using three recently developed TAFI assays that are independent of TAFI genotypes. These assays make it possible to determine not only intact TAFI levels, but also provide information on TAFI activation. In addition, we have measured functional TAFI activity. A limitation is the small size of our group of PAD patients. Only patients with a first clinical manifestation of peripheral arterial disease at young age were included. The statistically significant difference that we observed does not automatically mean that our findings are also of clinical relevance. Whether there is a role for TAFI in determining the outcome of PAD was not the aim of our study and needs to be studied in large, prospective studies. We selected our patients and controls from the same population (controls were friends of the patients), which eliminates part of the potential patient-control differences, but that remaining differences between patients and controls may contribute to our findings.

In conclusion, our study, although carried out in a small group of patients, shows for the first time that increased levels of TAFI are associated with risk of premature peripheral arterial disease.

Conflict of interest statement

None declared.

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